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Quantitative Changes in Cytomegalovirus DNAemia and Genetic Analysis of the UL97 and UL54 Genes in AIDS Patients Receiving Cidofovir Following Ganciclovir Therapy

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Five AIDS patients with cytomegalovirus (CMV) retinitis who had received ganciclovir (GCV) therapy were followed with serial blood sampling to detectchanges both in CMV load and in the genetic composition of genes UL97 and UL54 whilst receiving cidofovir (CDV) therapy. CDV neither reduced CMV load in blood nor prevented its quantitative resurgence during therapy. These effects were not explained by the initial presence or development of CDV-associated drug resistance mutations in UL54. In two patients, UL97 genotypic resistance to GCV involving either a L595S mutation or a deletion of amino acids 590–603 were present at the initiation of CDV and, in both patients, repopulation of CMV strains with wild-type UL97 sequences occurred during CDV therapy. These data are consistent with GCV-resistant strains containing UL97 mutations being less fit than their wild-type counterparts and so being able to persist only with the selective pressure of GCV. ***J. Med. Virol. 58:402–407, 1999.***

KEY WORDS: cytomegalovirus; ganciclovir; cidofovir; AIDS

INTRODUCTION

Cytomegalovirus (CMV) retinitis affects up to 40% of patients with AIDS. Current licensed therapies such as ganciclovir (GCV), foscarnet (FOS), and cidofovir (CDV) have been shown to slow the progression of CMV retinitis [Palestine et al., 1991; Spector et al., 1993; Anonymous, 1994; Lalezari et al., 1997; Bowen et al., 1998]. Following intravenous GCV induction therapy, a rapid reduction in urine and blood viral load occurs [Bowen et al., 1996]. The long-term use of GCV maintenance therapy has been associated with the development of GCV resistance, which has been mapped to the UL97 and UL54 (DNA polymerase) genes of CMV [reviewed in Drew et al., 1991; Chou et al., 1995; Biron, 1996; Crumpacker, 1996; Smith et al., 1997; Erice et al., 1997]. Recent data from our laboratory and others indicate that patients becoming CMV polymerase chain reaction (PCR) positive while receiving GCV maintenance therapy is strongly correlated with the development of UL97 mutations, which confer GCV resistance [Bowen et al., 1996, 1998]. Although CDV has been shown to reduce viral load in the urine and semen (Gilead studies GS 101 [Lalezari et al., 1995], 103 [Polis et al., 1995], 105 [Anonymous, 1997], and 106 [Lalezari et al., 1997]), CDV treatment frequently does not alter the qualitative presence of CMV in blood despite its clinical benefits in the eye [Anonymous, 1997; Lalezari et al, 1997]. Clinical isolates of CMV that are resistant to GCV and cross-resistant to CDV in vitro have been reported [Smith et al., 1997]. To assess the relationship between CMV viral load, genotypic changes in UL97 and UL54 and clinical outcome in patients undergoing CDV therapy, we have investigated intensively five patients with progressive CMV retinitis who received CDV therapy following prolonged exposure to ganciclovir.

MATERIALS AND METHODS

Study Population

Patients with CMV retinitis were recruited prospectively from September 1993 to a CMV viral load study

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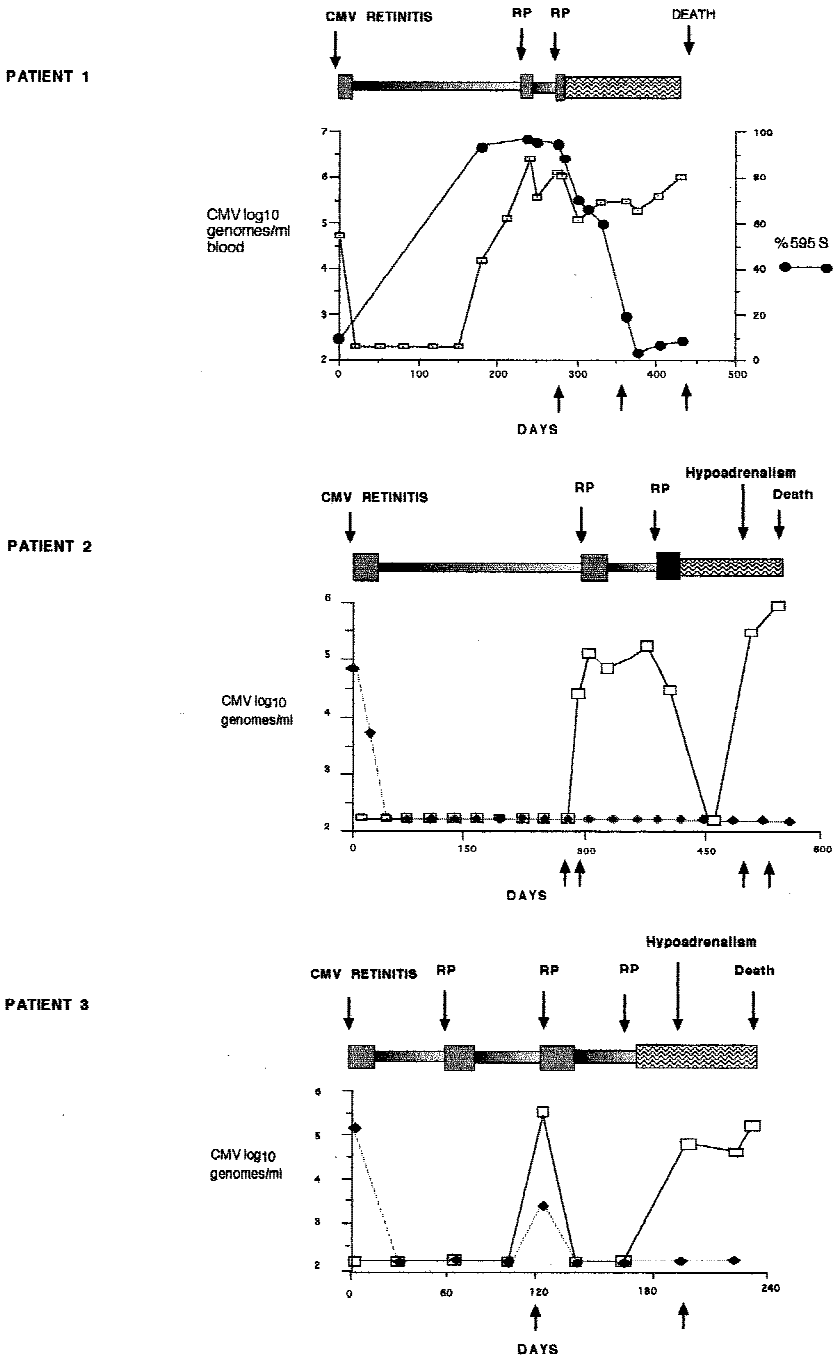

Fig. 1. Temporal fluctuations in viral load following the diagnosis of cytomegalovirus (CMV) retinitis in the five patients studied. Drug therapy and clinical outcomes are shown. The vertical arrows beneath the time axis indicate the times at which samples were analysed for UL54 mutations (see Table I). In patient 1, the results of the point mutation assay at codon 595 of UL97 are also shown.

[Bowen et al., 1996]. We studied five patients who received CDV for relapsing CMV retinitis (all of whom were enrolled in the GS 107 study [Lalezari et al., 1997]). These patients had been prescribed GCV for a mean of 222 days. Blood samples were collected at retinitis diagnosis, following induction therapy, and then monthly thereafter, with additional samples collected at times of retinitis progression or the development of other CMV-related disease. CMV disease was diagnosed according to the criteria outlined at the International CMV Workshop.

CMV PCR

DNA, extracted from whole blood using a commercial DNA extraction kit (Qiagen, UK), was subjected to PCR for CMV glycoprotein B (gB) (UL55) as described previously [Fox et al., 1992]. All samples qualitatively positive for gB were analysed further by a quantitative-

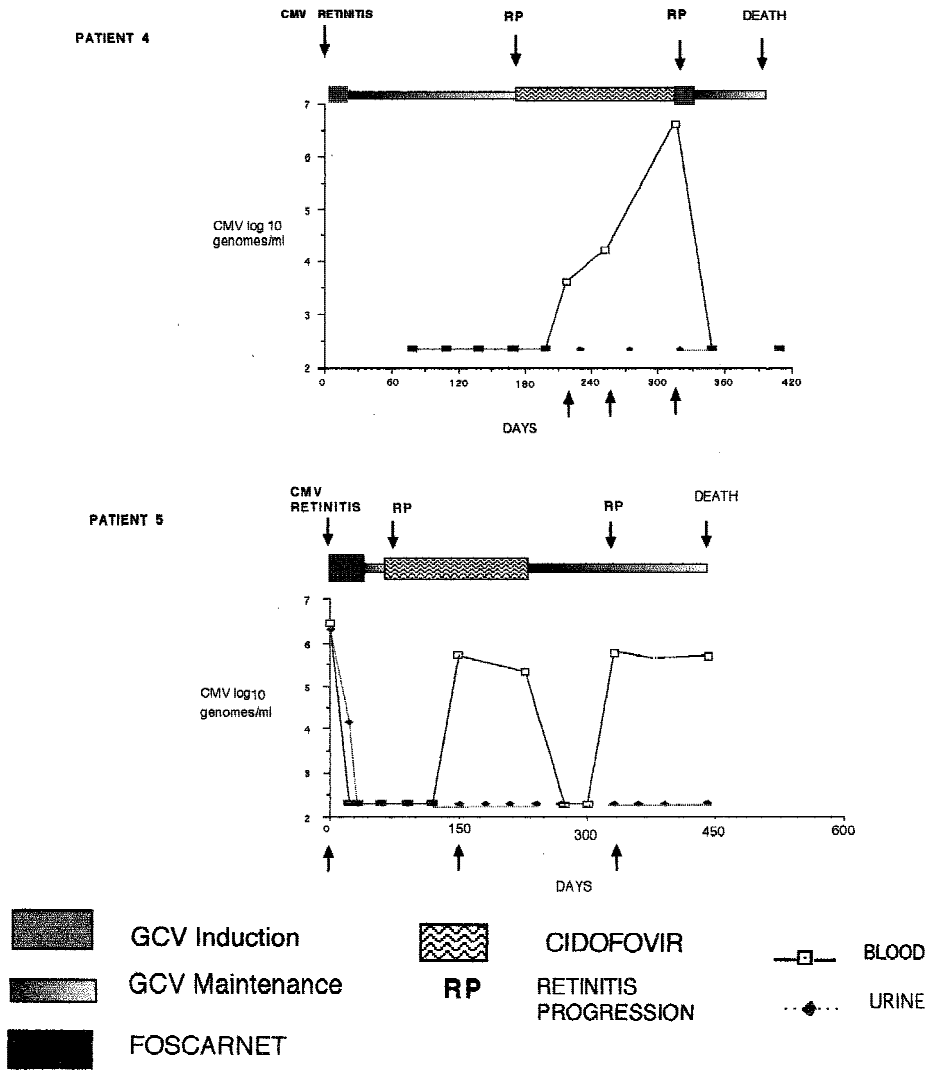

Fig. 1. (Continued).

competitive PCR to quantify the CMV load [Bowen et al., 1996].

Genotypic Analysis of the UL97 Gene

PCR-positive samples were re-amplified using primers specific for the UL97 gene. Primers and PCR conditions are described elsewhere [Bowen et al., 1997; Smith et al., 1997]. All UL97 positive samples were then analysed by a point mutation assay (PMA) to detect the six most common point mutations in UL97 known to confer GCV resistance to CMV (M460V, M460I, H520Q, A594V, L595S, and L595 F) [Bowen et al., 1997]. In addition, in all cases, direct DNA sequencing using conventional dideoxy methods was conducted on amplicons encompassing nucleotides 815–2172 of UL97 (non-nested PCR) or nucleotides 1205–1981 (nested PCR).

Genotypic Analysis of the UL54 Gene

CMV PCR-positive samples were re-amplified using primers specific for the DNA polymerase gene [Smith et al., 1997]. Single PCR products encompassing nucleotides 481–3089 of CMV DNA polymerase were generated using methods described previously [Smith et al., 1997]. If nested PCR was required to generate sufficient template for sequencing, the first PCR round described above was followed by a second PCR round to generate an internal fragment encompassing nucleotides 553–3034. Nucleotides 900–2700 (coding for amino acids 300–900) spanning the functional domains of the protein were sequenced using conventional dideoxy methods. Comparison of UL54 sequences were undertaken with AD169. However, common polymorphisms within UL54, which do not segregate with GCV resistance, have been reported elsewhere [Smith et al., 1997].

RESULTS

Modulations in CMV Viral Load During GCV and CDV Therapy

Of the five patients in this study, four were PCR positive in blood or urine at diagnosis of retinitis and

all four became PCR negative following 21 days of GCV induction therapy (Fig. 1). Patient 4 had been CMV positive in blood at another centre prior to enrolling in this study. Therefore, all five patients were CMV PCR negative in both blood and urine during the early stages of GCV maintenance therapy. All patients exhibited clinical progression of their retinitis whilst receiving GCV therapy and were therefore eligible for CDV therapy as part of the GS107 study. The viral load profiles, clinical course, and drug treatment of the five patients are shown in Figure 1. Patients 2, 3, 4, and 5, who were CMV PCR-negative on initiation of CDV therapy, became CMV PCR positive whilst receiving CDV therapy; patient 1 remained PCR positive with relatively minor changes in their viral load. Viral loads in excess of 5 $\log_{10}$ genomes/ml of blood were observed in all five patients during CDV therapy.

CMV Disease

Despite the appearance or persistence of a high CMV load while receiving CDV, retinitis remained clinically stable in four of the five patients. Two of these patients with stable retinitis required gluco- and mineralocorticoid replacement therapy for presumed CMV hypoadrenalism. However, there were no other cases of biopsy proven end-organ disease associated with the CMV DNAemia observed on CDV therapy.

UL97 Genotype

All 5 patients had wild-type UL97 gene sequences prior to starting ganciclovir. One patient (#1) developed an L595S mutation in UL97 as detected by the point mutation assay and confirmed by direct sequencing methods. The DNA extracts from samples from patient 2 would not bind consistently with the PMA probes and were therefore sequenced conventionally. A 42-bp deletion between bases 1767 and 1813 (coding for amino acids 590–603) was identified (Fig. 2). Interestingly, the UL97 mutations identified during GCV treatment in samples from Patients 1 and 2 were no longer detectable after CDV therapy by conventional sequencing methods and samples from Patient 1 showed a reducing prevalence of mutant by the PMA method in samples obtained after CDV therapy implicating the relatively rapid re-population of the viral pool with wild-type UL97 strains. The remaining three patients (#3, 4, and 5) had wild-type UL97 sequences throughout treatment.

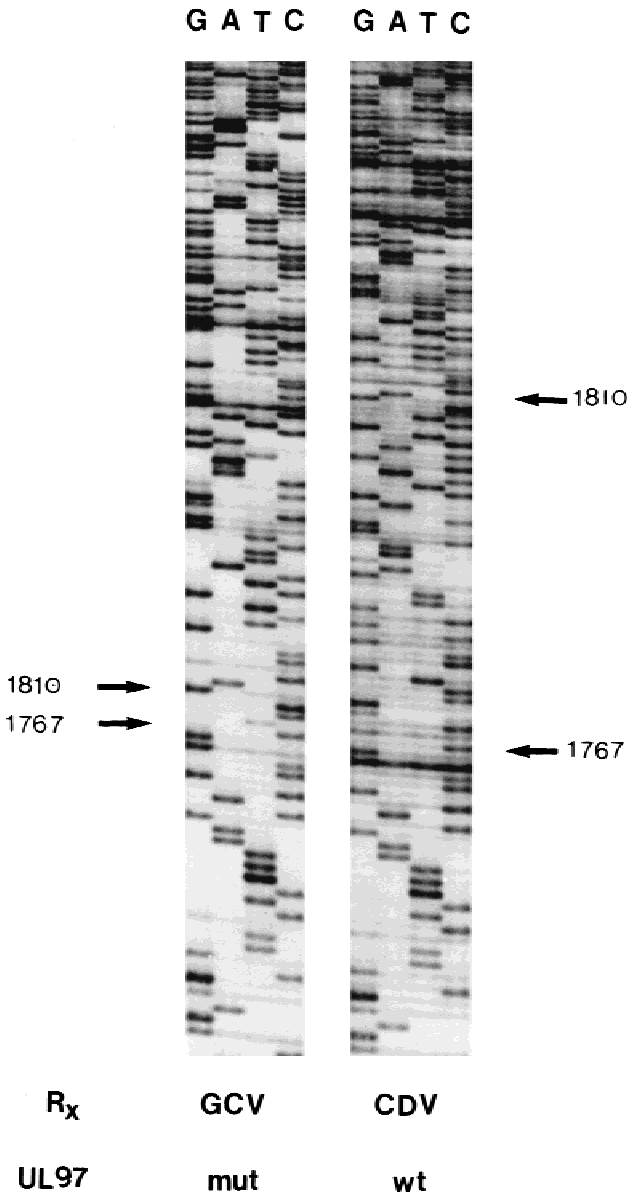

Fig. 2. Autoradiograph of a DNA sequencing gel encompassing the region between amino acids 590 and 603 of UL97 in patient 2. The deletion of 42 bp is observed in the sample obtained when the patient was treated with ganciclovir (GCV, left-hand lane). Following cidofovir (CDV) therapy, strains carrying the wild-type (wt) UL97 gene have re-emerged (right-hand lane). mut, mutation.

UL54 Mutations

To determine whether persistence of viral DNA during CDV therapy was associated with the development of mutations in DNA polymerase, a genotypic analysis was undertaken. Two patients (#1 and 3) showed wild-type CMV DNA polymerase sequence throughout therapy (Table I). While receiving GCV alone or GCV/foscarnet therapy, two patients (#2 and 5) developed mutations in conserved regions of UL54 [Smith et al., 1997]. Patient 2 developed a P522L mutation (50% mixture of wild-type and mutant), which was present in the conserved δ region C of UL54. Interestingly, this mutation was transient, as it became undetectable by standard sequencing methods after the patients had been taking CDV for a few months. Patient 5 also developed a mutation at codon 522 (P522S) as well as a cysteine-to-phenylalanine substitution at amino acid 830 (C830F), a highly conserved amino acid among the herpesviruses located in conserved region III; both of the mutations in this patient were present as 50% mixtures of wild-type and mutant amino acids. Patient #4 developed a V759M substitution while receiving CDV. This mutation is not within a conserved domain of poly-

TABLE I. Prevalence of UL97 and UL54 Mutations in Five Patients Prescribed Cidofovir for Progressive CMV Retinitis*

Patient no.	Days post-diagnosis[a]	Retinitis[b]	UL97 genotype	UL54 genotype
1	0	New Diagnosis	wt	—
	179	Relapsed	L595S	—
	274	Relapsed	L595S	S655L, N685S, A885T, N898D
	301	Stable	wt	—
	361	Stable	wt	S655L, N685S, A885T, N898D
	375	Stable	wt	—
	430	Stable	wt	S655L, N685S, A885T, N898D
2	0	New diagnosis	wt	—
	271	Stable	del 590–603	wt[c]
	294	Relapsed	del 590–603	50% P522L, S655L, N685S
	475	Stable	wt	50% S655L
	510	Stable	wt	50% N685S, A885T
3	120	Relapsed	wt	A885T, S897L
	210	Stable	wt	S655L, N685S, A885T, N898D
4	210	Stable	wt	S655L, N685S, A885T, N898D
	253	Stable	wt	—
	310	Relapsed	wt	S655L, N685S, V759M, A885T, N898D
5	0	New diagnosis	wt	—
	150	Stable	wt	wt
	320	Stable	wt	50% P522L, S655L, N685S 50% C830F, 50% G874R A885T, N898D

CMV, cytomegalovirus; wt, wild-type; del, deletion.
*Refer to Figure 1 for time on therapy and viral load values.
[a]Days from retinitis presentation.
[b]Clinical status of CMV retinitis.
[c]DNA sequence between aa522 and 812 was performed.

merase and the amino acid itself is not well conserved within the herpesviruses. Mutations in this region of polymerase have not been associated previously with CMV drug resistance. Interestingly, the codons associated with GCV resistance both disappeared following therapy with CDV. Polymorphisms in UL54 have been described in laboratory strains and in pre-therapy, drug-sensitive isolates (see Methods) and were observed at amino acids S655L, N685S, A885T, N898D, S897L, and G877R in samples from all five patients analysed in the present study.

DISCUSSION

This study illustrates the quantitative changes in the genotypic composition of the UL54 and UL97 genes of CMV in five patients with CMV retinitis following sequential treatment with GCV and CDV. Two patients developed UL97 mutations on GCV maintenance therapy that were associated with disease progression. In patient 1, a well-characterised mutation was present (L595S), whereas patient 2 exhibited a novel deletion not described previously in GCV-resistant strains of CMV. This deletion removed 14 amino acids (590–603) from UL97, a change that presumably rendered the virus less susceptible to GCV whilst still allowing CMV replication despite its location in a highly conserved, functional domain of the protein. This deletion encompassed the 591–594 deletion described in the prototypic GCV-resistant strain of CMV. Incorporation of the 590–603 deletion into a wild-type AD169 background is currently in progress to confirm its decreased ability to phosphylorate GCV. Interestingly, after these two patients were switched to CDV therapy, only wild-type UL97 sequences were detected by conventional sequence analyses. Also, the results of the point mutation assay illustrated that there was a gradual re-population of CMV strains containing wild-type UL97 sequence. This observation is consistent with strains harbouring UL97 mutations being less fit than wild-type virus in the absence of GCV and, because CDV does not require UL97 for activation, there is no longer a growth advantage for the UL97 mutant, hence the re-population with wild-type UL97 strains. Because the UL97 locus was almost homogenous for mutant virus at the initiation of CDV therapy, it is likely that strains carrying wild-type UL97 remaining in the population outcompeted the mutant strains. This situation is likely to be facilitated by the observation that AIDS patients frequently contain multiple CMV strains.

Sequence analyses of DNA polymerase from the five patients showed that the recrudescence or persistence of CMV DNAemia was unlikely to be due to the presence of CMV strains cross-resistant to GCV and CDV or due to the evolution of cidofovir resistance. One patient developed a new polymerase mutation (V759M) while receiving CDV, which has been shown by marker transfer not to confer GCV or CDV resistance. Interestingly, this patient showed a dramatic decrease in viral load upon subsequent GCV therapy, consistent with this mutation not conferring cross-resistance to GCV in vivo. Two patients (#2 and 3) developed mutations in conserved regions of polymerase (δ region C and region III) whilst on GCV therapy, which may play

a role in GCV resistance; in patient #5, these mutations were accompanied by a concomitant rise in viral load. Previous extensive genotypic analyses of GCV resistant clinical isolates would suggest that δ region C and region III may be "hot spots" for polymerase mutations that confer resistance to GCV and cross resistance to cidofovir in vitro [Smith et al., 1997]. In marker transfer experiments, the presence of mutation P522S in patient five was associated with a relatively modest (threefold) increase in the IC_{50} of CMV for GCV and CDV. This mutation developed whilst the patient was treated with GCV following a period of 120 days of CDV therapy. Virus sequences present after 65 days of CDV were wild type despite a viral load of $10^{5.6}$ genomes/ml blood.

In previous large clinical trials, CDV had been shown to delay retinitis progression. In the present intensive study of five patients, two patients were diagnosed with CMV adrenalitis on the basis of symptomatic improvement following mineralo-corticosteroid therapy. A previous comprehensive study showed that among patients with CMV retinitis who are also antigenemic, approximately 70% had a subnormal adrenocorticotrophin test half of whom were asymptomatic for their adrenal insufficiency [Hoshino et al., 1997]. Thus, CMV adrenalitis is a major problem in AIDS retinitis patients, irrespective of antiviral therapy, and is likely to occur following a resurgence of CMV viraemia. Clearly, CDV has been shown to be beneficial in the therapy of CMV retinitis. The metabolism of the drug in different tissues may be linked to its apparent differential effects on CMV replication in body compartments, for example, the retina versus the blood. It is therefore interesting to note that CDV is metabolised five- to eightfold less efficiently in whole blood cells as compared with numerous fibroblast and epithelial cell lines (unpublished data). Thus, the different pharmacologic properties of CDV as compared with GCV (and foscarnet), may explain differences in apparent efficacy at different body sites, and further studies comparing both virologic and clinical data are required to assess novel therapeutics.

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